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Inhibitory mechanism of pure curcumin on *Wilms' tumor 1* (*WT1*) gene expression through the PKCα signaling pathway in leukemic K562 cells

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ABSTRACT

The aim of this study was to investigate the inhibitory mechanism of pure curcumin on WT1 expression in leukemic K562 cells. Pure curcumin suppressed WT1 expression, independent of effects on protein degradation or WT1 mRNA stability. Chromatin immunoprecipitation and reporter gene assays indicate that pure curcumin treatment attenuates WT1 auto-regulation. Interestingly, PKCα inhibition mimicks the repressive effects of pure curcumin in K562 cells. Conversely, myristoylated PKCα over-expression increased WT1 expression and reversed the inhibitory effect of pure curcumin. Our study indicates that pure curcumin attenuates WT1 auto-regulatory function through inhibition of PKCα signaling in K562 cells.

1. Introduction

The *Wilms' tumor 1* (*WT1*) gene encodes a DNA-binding transcription factor essential for embryonal development [1–3]. High levels of WT1 are detected in most cases of chronic myelogeous leukemia (CML) in blast crisis and acute human leukemia, as well as leukemic cell lines. First discovered in 1990 and thought to function as a tumor suppressor, the WT1 transcription factor regulatory activity has been extensively studied to support a role for WT1 in oncogenesis [4].

Turmeric (*Curcuma longa* Linn) is one of the most popular medicinal herbs, due to its anti-inflammatory, antioxidant, anticarcinogen, anti-mutagen, and anticancer properties. The active constituents of turmeric are known as curcuminoids, yellow pigmented substances isolated from the rhizome of turmeric. Curcuminoids consist of three distinct components: curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcumin has strong anticancer and antitumor progression properties [5]. Nevertheless, many biological functions of curcumin result from its effects on PKC. For example, curcumin opposes the ability of phorbol esters to activate PKC in 3T3 cells [6], to promote tumor growth [7], and to activate the NF-κB and c-jun/AP-1 [8]. Moreover, the effects of curcumin are similar to the effects of PKC inhibitors: both curcumin and staurosporine inhibit the function of c-jun/AP-1 [8] and induce apoptosis in transformed cell lines [9].

Protein kinase C (PKC) is a family of serine/threonine protein kinases involved in regulating cell growth, differentiation, metabolism, and apoptosis [10]. Pure curcumin has been reported to affect multiple cell signaling pathways including the PKC signaling pathway [11–13]. Curcuminoids are a group of diet-derived agents that are being clinically evaluated as chemopreventive agents for several cancer types as well as leukemias [14,15]. Pure curcumin was recently shown to repress *WT1* gene expression in both primary and leukemic cells [16].

Our current study indicates that one anti-proliferative mechanism of pure curcumin is through attenuation of WT1 auto-regulation by inhibiting PKCα signaling.

2. Materials and methods

2.1. Cell culture

Human leukemic cell lines K562 (kindly provided by Dr. Chaisuree Supawilai, Research Institute for Health Sciences, Chiang Mai, Thailand) was cultured in RPMI 1640 (Invitrogen, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, CA, USA) and incubated at 37 °C.

Eukaryotic expression vector pcDNA3.1 carrying the cDNA for WT1 [17AA(+)KTS(+)] or WT1(+/+), WT1 [(17AA(+)KTS(−)] or

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WT1 (+/−), WT1 [17AA(−)KTS(+)] or WT1 (−/+), or WT1 [17AA(−)KTS(−)] or WT1 −/− were kindly provided by Prof. Dr. Haruo Sugiyama and Assoc. Prof. Dr. Yusuke Oji, Osaka University Graduate School of Medicines, Osaka, Japan [17].

2.2. Extraction and isolation of pure curcumin

Pure curcumin was purified from turmeric powder using column chromatography. Pure curcumin extraction and isolation were performed as previously described [18,19].

2.3. Proliferation and viability assay

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma–Aldrich, MO, USA) as described previously [20]. The reaction is catalyzed by mitochondrial succinate dehydrogenase and requires NADH, which must be supplied by living cells.

Cell proliferation was measured by the trypan blue exclusion method. Cells were transfected with pcDNA3.1 or four different WT1 isoforms for 48 h and treated with 25 μM pure curcumin or 0.04% DMSO for 48 h. Then cells and 0.4% trypan blue dye were mixed and counted using a light microscope. All experiments were performed in triplicate.

2.4. RNA isolation, reverse transcription and real-time PCR analysis

The K562 cells were treated with pure curcumin and then harvested and RNA was isolated using RNeasy® Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol. The cDNA was synthesized from 5 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, CA, USA) with random hexamer primers according to the manufacture's instruction. Real-time analysis was carried out with a BioRad iCycler iQ real Time PCR instrument with TaqMan probe-based chemistry for WT1 (Hs01103754) and GAPDH (Hs99999905) (Applied Biosystem, CA, USA) using Two Step RT qPCR Master Mix (Applied Biosystem, CA, USA).

2.5. Preparation of membrane fractions

To detect the activated (membrane associated) fraction of PKCα in treated K562 cells, cells were washed twice with PBS and then washed with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT). The cell pellets were resuspended in buffer A containing 0.1% Nonidet P40 and homogenized using a conical homogenizer and then incubated on ice for 30 min. After 30 min, the cell homogenates were centrifuged at 2900×*g* for 20 min. The supernatant was collected and centrifuged at 100 000×*g*, 4 °C for 1 h. The supernatant of this spin was collected as the cytosolic fraction. The pellets (considered the membrane fraction) were resuspended in RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 0.5 mM EDTA) containing protease inhibitors and then the protein concentrations were measured by Lowry's method (*DC* Protein Assay Kit; BioRad, CA, USA).

2.6. Preparation of whole lysate protein and immunoblotting

The treated cells were washed twice with PBS and lysed with cold RIPA buffer containing protease inhibitors and centrifuged. Fifty micrograms of crude protein or 30 μg of membrane protein fraction were separated by SDS–PAGE, transferred to nitrocellulose membrane and blocked in 5% non-fat milk. Membranes were probed with anti-WT1 antibody (C-19, Santa Cruz, CA, USA), anti PKC isotype antibody (Cell Signaling Technology, CA, USA) or anti GAPDH antibody (Santa Cruz, CA, USA). The following secondary antibodies were used: goat-anti-rabbit-horseradish peroxidase, goat-anti-mouse-horseradish peroxidase or goat-anti-rat-horseradish peroxidase (Invitrogen, CA, USA). Peroxidase activity was detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Miami, USA) and an Alpha Innotech gel imaging system (Cell Biosciences Inc., CA, USA). Densitometry was performed using Alpha Innotech software.

2.7. Protein and mRNA half life analysis

K562 cells were incubated with 50 μg/ml cycloheximide (CHX) in the presence or absence of 15 μM pure curcumin. For the proteasome inhibitor assay, cells were incubated with or without 5 μM MG132 or 10 μM (−)-Epigallocatechin gallate (EGCG) (Sigma–Aldrich, MO, USA) or 10 μM Lactacystin (TRC, Ontario, Canada) in the presence or absence of 15 μM pure curcumin for 12 h. K562 mRNA stability was assessed with 4 μM actinomycin D in the presence and absence of 15 μM pure curcumin. For PKCα inhibitor treatment, K562 cells were incubated with 0.0084 μM GF109203x (IC_{50}).

2.8. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay previously described [21], was used to detect interaction between transcription factors and the proximal WT1 promoter. K562 cells were treated with 15 μM pure curcumin or vehicle control for 24 h and crosslinked with 1% formaldehyde. For the immunoprecipitation, anti-WT1 (C-19) antibody, anti-Sp1 antibody (Santa Cruz, CA, USA) or anti mouse IgG antibody (MP Biomedical, OH, USA) were added and incubated overnight at 4 °C. The chromatin immunoprecipitated DNA was amplified by standard PCR or ChIP-qPCR using SYBR Green Master Mix (BioRad, CA, USA). WT1 primer: 5′-CCTGAACG-GACTCTCCAGTG-3′ (forward) and 5′-CGCTGCCTTGAACTCCTTAC-3′ (reverse). The WT1 promoter sequences were referenced from GenBank™ Accession No. U77682 and based on DNA sequence published in Fraizer et al. [22].

2.9. Transient transfections

DNA plasmid vector containing Myr.PKCα (Addgene, MA, USA) was transfected into K562 cells by Lipofectamine™ LTX with Plus™ reagent (Invitrogen, CA, USA). After 48 h, the transfected cells were treated with 15 μM pure curcumin for 24 h.

2.10. Promoter luciferase-reporter assays

K562 cells were plated in 12-well dish format and transfected the next day with 1 μg of the proximal WT1 pGL3 Basic luciferase reporter construct (kindly provided by Prof. Dr. Takashi Murate) [23] and 80 ng beta-galactosidase pCMX vector by Lipofectamine™ LTX with Plus™ reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After 6 h, the cells were treated with pure curcumin for 24 h, lysed in Reporter Lysis Buffer and the firefly luciferase activity measured using the Luciferase Assay Kit (Promega, WI, USA) on a Veritas 96 Microplate Luminometer (Promega, WI, USA). The firefly luciferase was normalized with β-galactosidase activity to control for transfection efficiency. For the mutant pGL3 construct, core nucleotides of the putative WT1 binding site in the promoter were altered by Quick-Change Site Mutagenesis Kit (Invitrogen, CA, USA) using PCR primers for WT1 consensus sequence: −50 to −39 GTGTGGGAGCC [24] was mutated to *ATATGATATCA*.

2.11. Statistical analysis

Statistical analysis was performed with SPSS software (version 10.0). All experiments were performed in duplicate and repeated

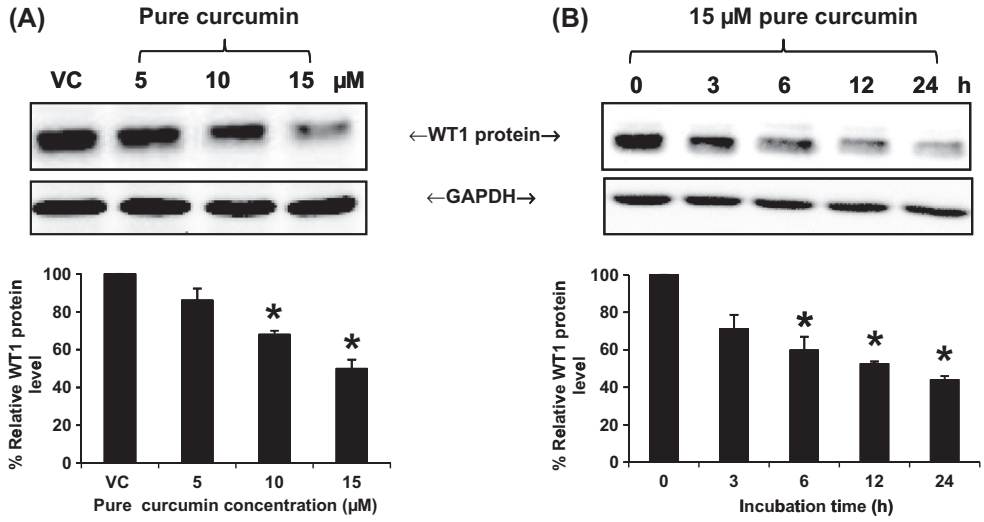

Fig. 1. Effect of pure curcumin on *WT1* gene expression in K562 cells. The level of WT1 protein expression was assessed by immunoblotting after treatment with various doses of pure curcumin for 24 h, shown in (A). Time course with 15 μM pure curcumin assayed at 0, 3, 6, 12, and 24 h, shown in (B). GAPDH was used as loading control. Densitometry was used to quantify the protein levels and graphed as the percentage of vehicle control. Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

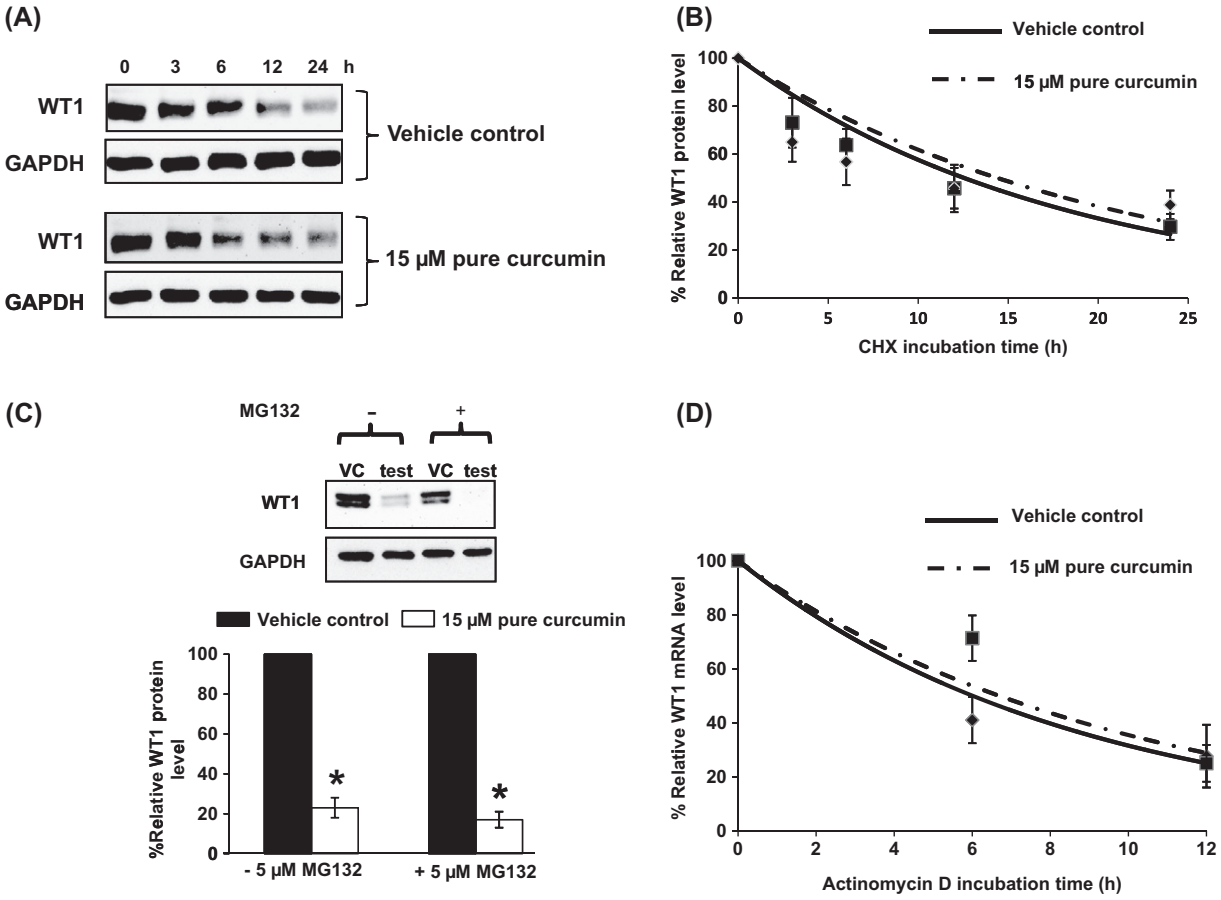

Fig. 2. Effect of pure curcumin on WT1 protein and mRNA stability. To investigate the effect of pure curcumin on protein stability, K562 cells were treated with 50 μg/ml cycloheximide in the presence or absence of 15 μM pure curcumin for 0, 3, 6, 12, and 24 h and assessed by immunoblotting, as shown in (A). WT1 protein levels from three independent experiments were quantified and shown in (B). (C) The effect of 15 μM pure curcumin on WT1 protein levels was examined in the presence or absence of 5 μM MG132. GAPDH was used as loading control. (D) K562 cells were treated with 4 μM actinomycin D in the presence or absence of 15 μM pure curcumin for 0, 6, and 12 h and WT1 mRNA was assayed by Taqman qRT-PCR. GAPDH was used as an internal control. Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

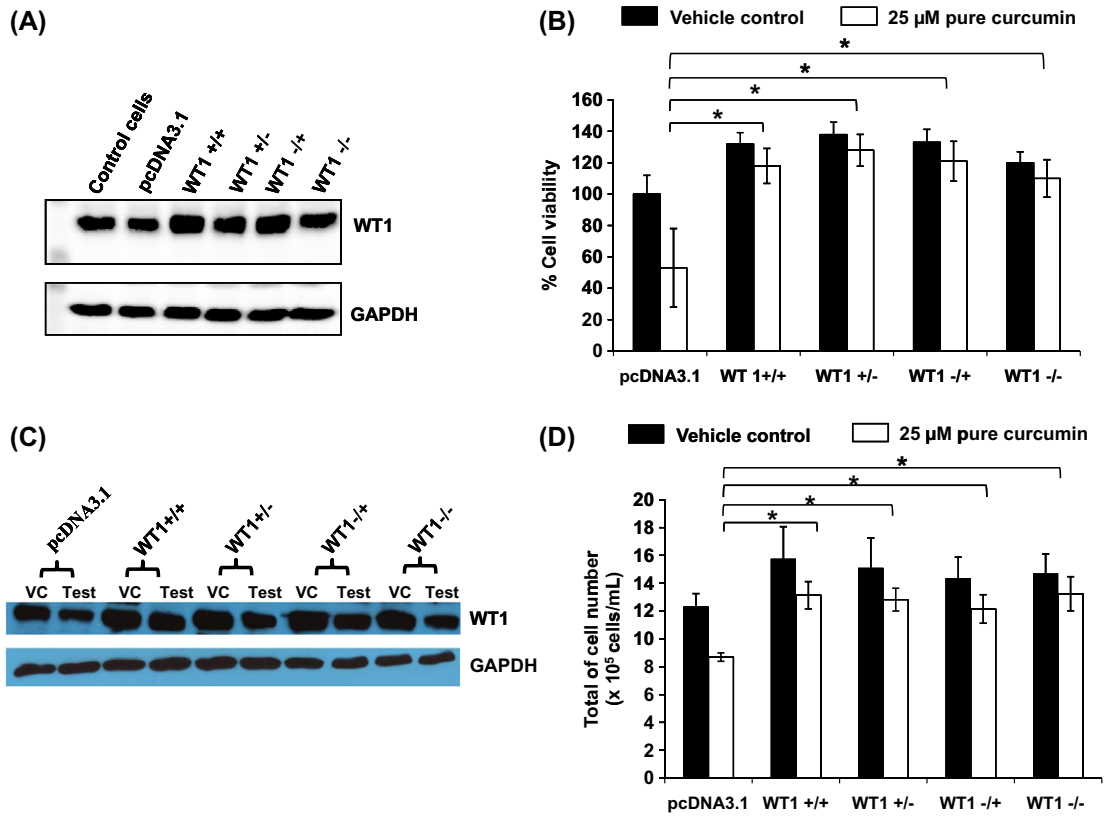

Fig. 3. Effect of WT1 isoform over-expression on pure curcumin activity. K562 cells were transiently transfected with different isoforms: WT1 +/+, WT1 +/−, WT1 −/+, WT1 −/−, or the pcDNA 3.1 vector for 48 h followed by treatment with 25 μM pure curcumin for 48 h. (A) Immunoblot analysis of WT1 isoform expression in the transfected K562 cells. GAPDH was used as a loading control. (B) Cell viability of K562 cells was measured with the MTT assay. (C) Immunoblot analysis of WT1 isoforms after treatment with 25 μM pure curcumin. (D) K562 total cell number was assayed by the trypan blue exclusion method. Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

at least three times. The data were expressed as the mean ± standard error of sample mean (S.E.M.). Statistical differences between the means were tested by one-way ANOVA. Probability values $P < 0.05$ were considered significant.

3. Results

3.1. Effect of pure curcumin on WT1 gene expression is not through protein or mRNA degradation pathways

The pure curcumin derivative of curcumunoids was previously shown to suppress the WT1 transcription factor mRNA and protein levels in several leukemic cell lines [16]. We first confirmed WT1 down-regulation by pure curcumin in a dose response and time course in K562 cells for this study (Fig. 1A and B). K562 cells were treated with pure curcumin at 15 μM (IC_{20}) or 25 μM (IC_{50}) for 24 h, or as indicated. To determine whether pure curcumin has any effect on WT1 protein stability, we performed a time course experiment with the protein synthesis inhibitor, cyclohexamide (CHX), to determine the half life of WT1 protein. The half life of WT1 protein treated with vehicle control at 12.6 h was not significantly different compared to cells treated with 15 μM pure curcumin at 14.4 h (Fig. 2A and B). In addition, we treated K562 cells with pure curcumin in the presence or absence of the proteasome inhibitors MG132, (−)-Epigallocatechin gallate (EGCG), and Lactacystin to determine whether proteasome inhibition would interfere with the repressive effect of pure curcumin on WT1 (Fig. 2C and Supplementary Fig. 1). WT1 protein expression decreased similarly after treatment with pure curcumin in the presence or absence of MG132 or EGCG or Lactacystin, indicating that the pure curcumin-mediated down-regulation of WT1 protein is not through proteasomal degradation pathways. Since pure curcumin has been shown to decrease WT1 mRNA levels [20], we examined whether WT1 mRNA stability was affected. K562 cells were treated with 4 μM actinomycin D and assayed over 12 h in the presence or absence of pure curcumin. The stability of WT1 mRNA was unchanged between vehicle-treated and pure curcumin-treated K562 cells (Fig. 2D). Together these results demonstrate that the down-regulation of *WT1* gene expression by pure curcumin was independent of protein or mRNA degradation pathways.

3.2. WT1 over-expression reverses the effect of pure curcumin activity

WT1 mRNA undergoes alternative splicing at two sites resulting in four mRNA species and protein products [25]; WT1 +/+, WT1 +/−, WT1 −/+, and WT1 −/− [22]. Since pure curcumin inhibits cell growth and represses WT1 expression, we sought to determine whether the two are linked. For this experiment, we determined whether we could reverse the anti-proliferative effects of pure curcumin by over-expressing exogenous WT1 in K562 cells. The WT1 isoform constructs were transfected into K562 cells and WT1 protein over-expression relative to vector control was confirmed by immunoblotting of lysates collected at 48 h after treatment with 25 μM pure curcumin (IC_{50}), as shown in Fig. 3A and C. Next, the anti-proliferative effect of pure curcumin on K562 cells transfected with WT1 isoforms was assayed by measurement of cell viability and proliferation using the MTT assay and trypan blue exclusion method. The over-expression of all four WT1 isoforms in K562 showed resistance to the anti-proliferative effects of pure curcumin activity when compared to transfected vector control

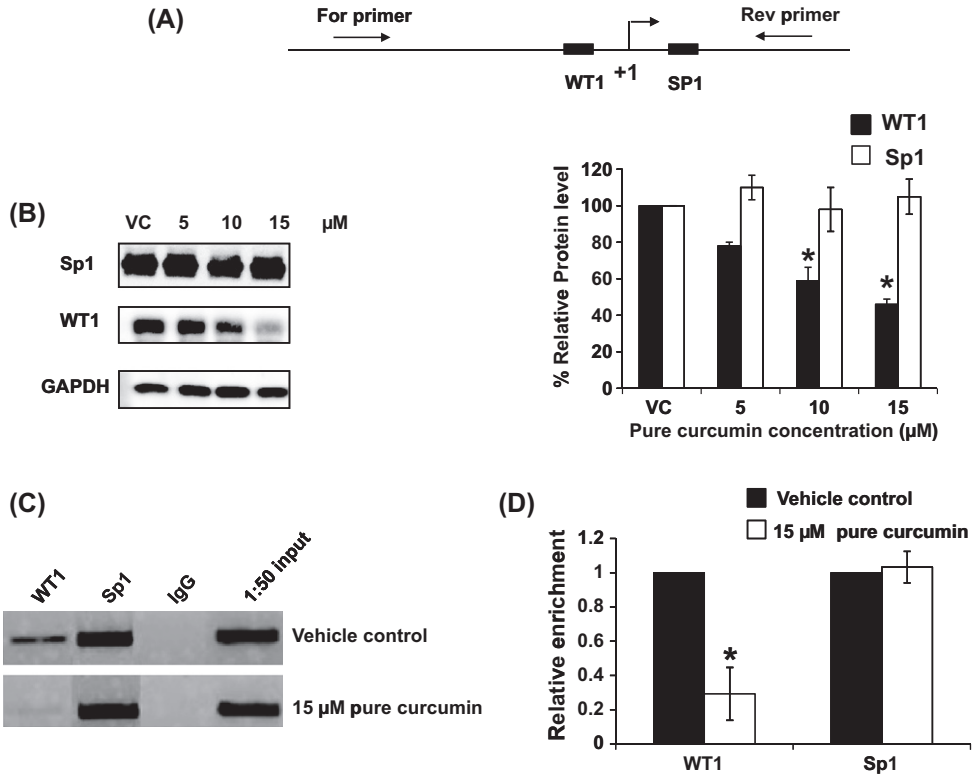

Fig. 4. Pure curcumin treatment attenuated WT1 but not Sp1 binding to the proximal WT1 promoter. (A) Schematic showing the WT1 proximal promoter and location of ChIP primers relative to the furthest upstream TSS (full sequence shown in Supplementary data [22]). (B) K562 cells were treated with 5, 10, and 15 μM pure curcumin or vehicle control for 24 h and lysates immunoblotted for WT1, Sp1, with GAPDH as a loading control. Immunoblot shown left and densitometry graphed as percent of vehicle-treatment (right panel). (C) K562 cells were treated with 15 μM pure curcumin for 24 h and lysates were immunoprecipitated as indicated for WT1, Sp1 or IgG control. Immunoprecipitates and 1:50 dilution of input were assayed by standard PCR for binding to the WT1 proximal promoter (see schematic). (D) The WT1 and Sp1 immunoprecipitated lysates from pure curcumin or vehicle control treatment were analyzed by SYBR green qPCR and graphed as relative DNA enrichment over 1:50 input as percent of vehicle-treatment. Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

(Fig. 3B and D). Importantly, ectopic WT1 protein expression reversed the anti-proliferative activity of pure curcumin. This finding provides key insight into the growth inhibitory mechanisms of pure curcumin. Our result also demonstrates the transcriptional redundancy of WT1 isoforms and their regulatory function in cell proliferation.

3.3. Pure curcumin attenuates WT1 auto-regulation

The WT1 promoter has been extensively analyzed [22] and shown to auto-regulate itself by the WT1 transcription factor [24], and by other transcription factors including GATA1 [23,26] and Sp1 [27]. WT1 also regulates immunosuppressant cytokine interleukin-10 (IL-10) gene [28]. K562 cells were treated with various doses of pure curcumin for 24 h and immunoblotted for WT1 and Sp1 protein levels. The pure curcumin concentrations of 10 and 15 μM showed down-regulation of WT1 protein but not Sp1 protein, demonstrating specificity (Fig. 4B). Next, the effect of pure curcumin on the interaction between WT1 and Sp1 transcription factors and the WT1 proximal promoter was investigated by chromatin immunoprecipitation assay. Pure curcumin prevented binding of WT1 to the region containing a WT1 consensus binding site in the proximal promoter (see schematic in Fig. 4A; promoter sequence [22]) as shown by standard PCR (Fig. 4C) or ChIP qPCR (Fig. 4D). The pure curcumin treatment had no effect on the binding of Sp1 (Fig. 4C and D). The inhibition of WT1-DNA binding but not Sp1 binding suggests that pure curcumin depletes WT1 through a loss of auto-regulatory function. In other words, pure curcumin disrupts a feed-forward loop in which WT1 promotes its own expression.

The WT1 ChIP assay demonstrated that pure curcumin abrogates WT1 transcription factor binding to the WT1 proximal promoter. To further evaluate the effect of pure curcumin on the WT1 promoter auto-regulation we obtained a luciferase reporter construct for the minimal proximal WT1 promoter sequence required for maximum response in K562 leukemia cells [23]. There are several potential WT1 and Sp1 consensus sites within the WT1 proximal promoter [22]. The WT1 (−50 to −39) and Sp1 (−224 to −203) consensus binding sites included within the 301 bp reporter construct are indicated (see schematic of sequence; Supplementary information). Transfection of the 301 bp construct into K562 cells demonstrated high luciferase activity with vehicle-control treated cells and a diminished response from pure curcumin treated K562 cells (Fig. 5A). To determine if the luciferase activity was driven by WT1 protein binding to the proximal promoter, the WT1 binding site located at −50 to −39 was mutated. Importantly, the luciferase construct containing the mutated WT1 binding site in the proximal WT1 promoter was much less responsive when transfected into K562 cells (Fig. 5B) suggesting WT1 binding to the proximal promoter was driving firefly luciferase expression. The WT1 binding site included within the luciferase reporter construct and the ChIP-qPCR amplified region suggest that the inhibitory effect of pure curcumin on WT1 auto-regulation is mediated at least in part through the upstream WT1 binding site located at position −50 to −39 in the WT1 proximal promoter.

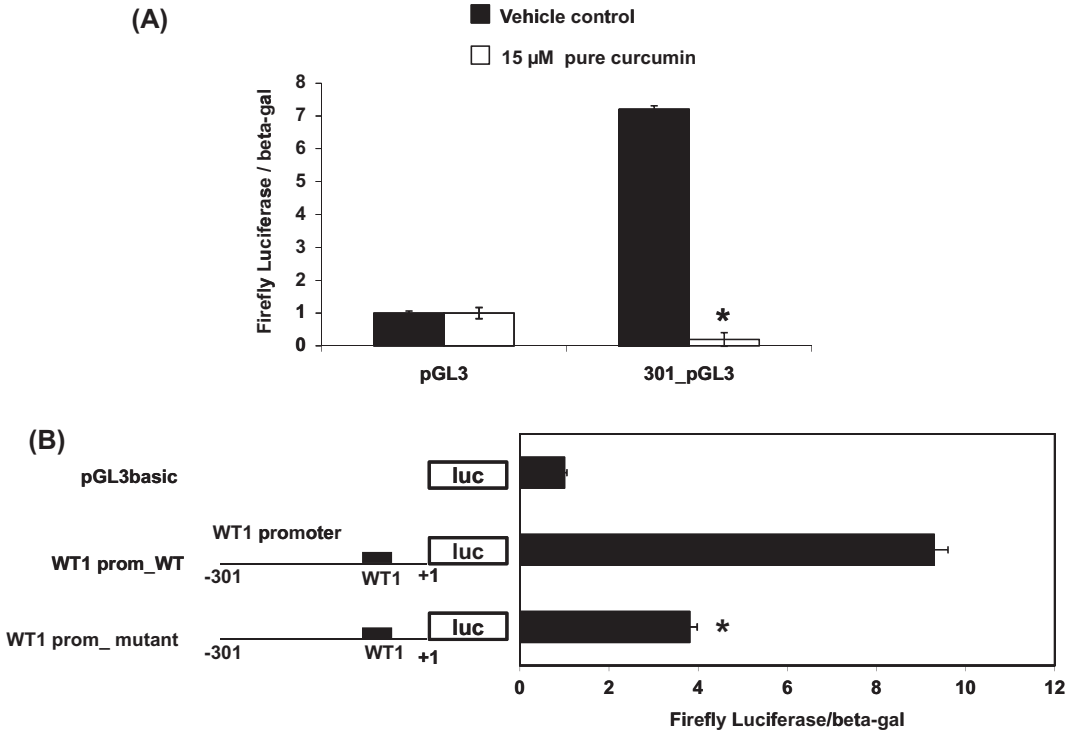

Fig. 5. WT1 promoter reporter activity is repressed by pure curcumin. (A) K562 cells were transfected with the pGL3_basic luciferase reporter vector containing 301 bp of the WT1 proximal promoter followed by 15 μM pure curcumin or vehicle treatment for 24 h. The firefly luciferase and beta-galactosidase activity were assayed and relative activity graphed compared to the pGL3 basic vector. (B) Site directed mutagenesis of the WT1 consensus sequence (−50 to −39) abrogated the WT1 promoter activity compared to the wild type WT1 promoter construct (301 bp WT1). Mutant or WT1 constructs were transfected for 24 h and then assayed for firefly luciferase and beta-galactosidase activities. Experiments were performed a minimum of three times and representative graph is shown. Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

3.4. Effect of pure curcumin on WT1 through the PKCα cell signaling pathway

Pure curcumin has been shown to inhibit PKC signaling and to inhibit WT1 expression in independent studies [11,16]. We next investigated whether the down-regulation of *WT1* gene expression by pure curcumin is through an inhibition of the PKCα signaling pathway. Preliminary data from phospho-kinase arrays (R&D System, MN, USA) probed with K562 lysates indicated decreased phospho-c-Jun and phosho-JNKpan (PKC targets) from pure curcumin treated compared to control-treated cells (Supplementary Fig. 2). To determine if PKCα is actively functioning at the cell membrane in K562 cells, we prepared cell membrane protein fractions and immunoblotted for PKCα or the membrane protein, caveolin 3, in K562 cells treated with pure curcumin or vehicle control. The immunoblot shows 15 μM pure curcumin significantly decreased PKCα occupancy at the membrane but had no effect on caveolin 3 expression (Fig. 6A).

To test whether inhibition of PKCα mimics pure curcumin treatment with respect to WT1 expression, we assayed WT1 protein levels after treatment with the PKCα protein kinase inhibitor (GF109203x) at several time points in K562 cells. The immunoblot shown in Fig. 6B indicates inhibition of PKCα activity decreased WT1protein after 3 h of GF109203x treatment and the level of protein expression remained decreased at 5 and 7 h (Fig. 6B).

3.5. PKCα activation up-regulates WT1 expression and reverses pure curcumin inhibition of WT1 expression in K562 cells

We next investigated whether PKCα activation could induce an increase in endogenous WT1 protein in K562 cells and reverse the pure curcumin effect on WT1 expression. K562 cells were transfected with Myr.PKCα vector or control vector for 48 h and treated with or without 15 μM pure curcumin for 24 h. The Myr.PKCα transfected K562 cells demonstrated an up-regulation of endogenous WT1 protein expression when compared to control transfected cells. The control transfected K562 treated with pure curcumin showed down-regulated endogenous WT1 protein as expected, and duplicated our previous results shown in Fig. 1A. In contrast, the Myr.PKCα transfected K562 cells increased endogenous WT1 expression and reversed the pure curcumin inhibitory effect on WT1 (Fig. 6C). In other words, in the presence of activated PKCα, pure curcumin was unable to suppress WT1 expression. This result provides a novel functional link between pure curcumin-mediated inhibition of PKCα and pure curcumin-mediated repression of WT1.

4. Discussion

Expression of the *Wilms' tumor 1* gene product has been shown to increase 1000–10 000 fold in leukemic cells [29,30], suggesting that the *WT1* gene may play an important role in oncogenesis. In anticancer drug research, dietary and/or medicinal plants, such as turmeric, ginger, garlic, chili, and pepper are becoming popular as chemotherapeutic and/or chemopreventative drugs. Curcumin is the main active ingredient of turmeric, a traditional herbal medicine and food of south Asia. Curcumin has been reported to affect multiple cell signaling pathways [31], and recent studies indicate curcumin inhibits WT1 expression in leukemic cell lines [16] and PANC-1 pancreatic cancer cells [32].

Previous work showed pure curcumin decreased WT1 mRNA and WT1 protein levels in several human leukemic cell lines

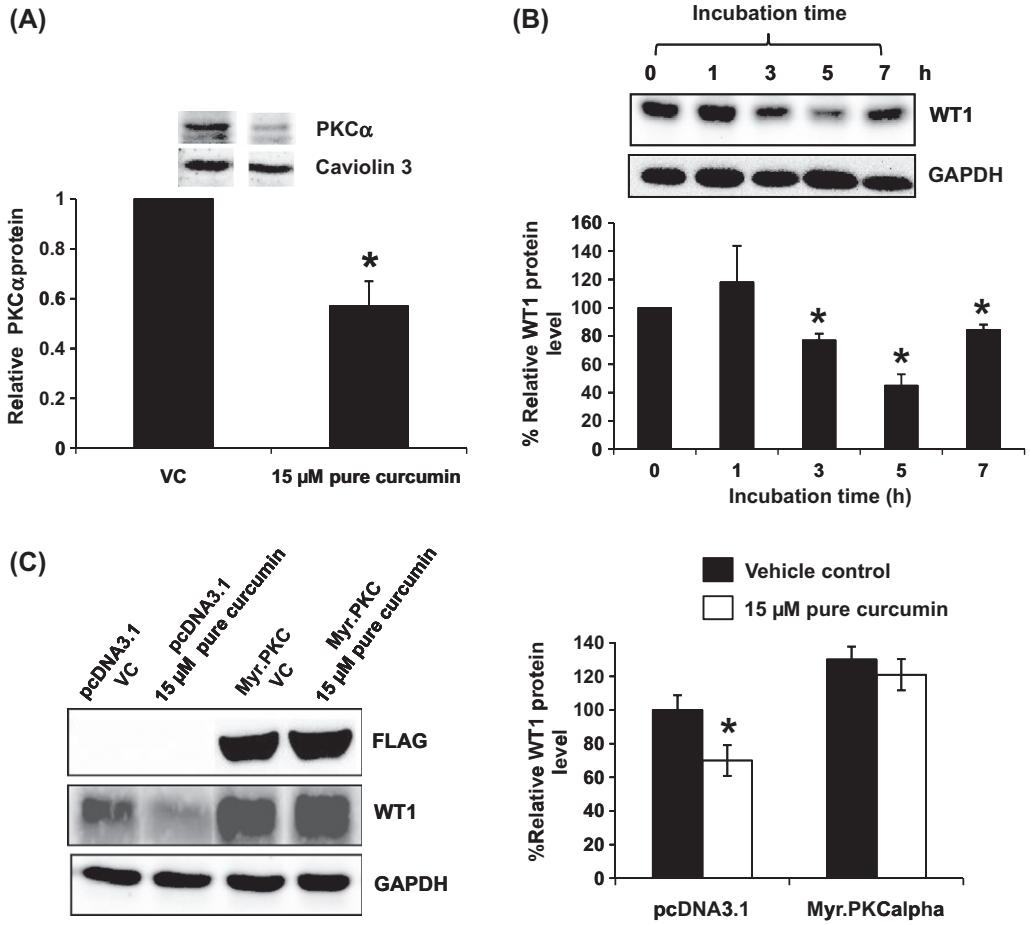

Fig. 6. The effect of pure curcumin on PKC signaling. (A) K562 cells were treated with 15 μM pure curcumin for 24 h and the membrane fraction collected and immunoblotted for levels of PKCα or the membrane protein, caveolin 3. (B) The effect of PKCα inhibitor on WT1 expression in K562 cells assayed after treatment with 0.0084 μM GF109203x (IC_{50}, PKCα inhibitor) at various time points. Immunoblot detection of the WT1 or GAPDH protein levels are shown (top) and densitometry graphed below. (C) K562 cells were transiently transfected with vector control or Flag-tagged Myr.PKCα. Cells were then treated with vehicle control or 15 μM pure curcumin. Lysates were immunoblotted for Flag (Myr.PKCα), WT1 or GAPDH loading control. Densitometry graphed as percentage of control treatment (right panel). Data are the mean value ± S.E.M. of three independent experiments. Asterisks (∗) denote values that were significantly different from the vehicle control ($P < 0.05$).

although the mechanisms were not explored [16]. This current study demonstrates that the pure curcumin-mediated down-regulation of WT1 was not the result of protein or mRNA degradation processes in the K562 leukemic cell line. Rather, both Chromatin immunoprecipitation and luciferase reporter assays indicate that pure curcumin interferes with WT1 binding to its DNA consensus site at the proximal promoter of the *WT1* gene, nullifying WT1's positive auto-regulatory role. Further reporter assays in K562 cells showed mutation of the DNA consensus site in the proximal promoter of the *WT1* gene attenuated WT1-binding and abolished reporter activity. Together these results demonstrated pure curcumin interferes with WT1 auto-regulatory activity, resulting in down-regulation of WT1 expression. Since ectopic over-expression of WT1 prevents growth inhibition by pure curcumin (Fig. 3), these results demonstrate that repression of WT1 is an essential mechanism by which pure curcumin inhibits tumor cell growth.

Several studies have shown that curcumin affects multiple signaling pathways, including protein kinase C [13,31,33,34]. We tested whether the pure curcumin inhibitory effect on *WT1* gene expression is mediated through the PKC signaling pathway. Treatment of K562 cells with the PKC inhibitor, GF109203x, down-regulated endogenous WT1 expression in K562 cells, mimicking the results obtained with pure curcumin. In addition, chronic activation of PKC by expression of Myr.PKCα demonstrated that PKCα activation rescued the pure curcumin inhibitory effect on WT1 expression.

Our results indicate that pure curcumin interferes with PKC signaling and WT1 expression and that the two are functionally linked. Both pure curcumin and PKC inhibitor treatment down-regulated WT1 expression, indicating that WT1 is downstream of the pure curcumin inhibitory effect on PKC signaling. Over-expression of Myr.PKCα increased WT1 protein levels and rescued the inhibitory effect of pure curcumin on WT1 expression. Transcription factor DNA binding is commonly regulated by phosphorylation. Early in vitro work on WT1 regulation reported that PKC phosphorylates WT1 at one of several potential sites, inhibiting DNA binding [35]. Our results suggest that PKCα phosphorylation may also enhance WT1 DNA binding. Interestingly, PKCα and pure curcumin have been shown to physically interact [13]. The mechanism of how PKCα positively regulates WT1 expression in K562 cells will require further investigation.

In conclusion, our results indicate the effect of pure curcumin on *WT1* gene expression in the leukemic K562 cell line was mediated through PKCα signaling upstream of the WT1 transcription factor auto-regulatory function. As a result, pure curcumin affected the WT1 protein-promoter binding and the decrease of WT1 mRNA and protein levels in K562 cells contributed to the pure curcumin anti-proliferative effect. This novel mechanistic knowledge of how pure curcumin effects WT1 transcriptional function in

leukemic cells may be useful in the future development of therapeutic approaches for leukemic patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.043.

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